

Acetic acid

UV-method

for the determination of acetic acid in foodstuffs and other materials

Cat. No. 10 148 261 035

Test-Combination for 3 × 11 determinations

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

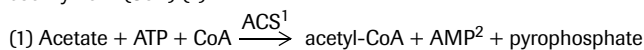
For *in vitro* use only

Store at 2-8°C

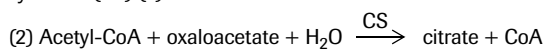
For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

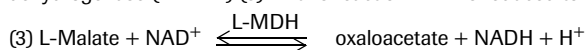
Acetic acid (acetate) is converted to acetyl-CoA in the presence of the enzyme acetyl-CoA synthetase (ACS)¹, adenosine-5'-triphosphate (ATP) and coenzyme A (CoA) (1).



Acetyl-CoA reacts with oxaloacetate to citrate in the presence of citrate synthase (CS) (2).



The oxaloacetate required for reaction (2) is formed from L-malate and nicotinamide-adenine dinucleotide (NAD) in the presence of L-malate dehydrogenase (L-MDH) (3). In this reaction NAD is reduced to NADH.



The determination is based on the formation of NADH measured by the increase in light absorbance at 340, 334 or 365 nm. Because of the equilibrium of the preceding indicator reaction, the amount of NADH formed is not linearly (directly) proportional to the acetic acid concentration (for calculations, see below).

The Test-Combination contains

1. Bottle 1 with approx. 33 ml solution, consisting of: triethanolamine buffer, pH approx. 8.4; L-malic acid, approx. 134 mg; magnesium chloride x 6 H₂O, approx. 67 mg
2. Bottle 2 with approx. 280 mg lyophilizate, consisting of: ATP, approx. 175 mg; CoA, approx. 18 mg; NAD, approx. 86 mg
3. Bottle 3 with approx. 0.4 ml suspension, consisting of: L-malate dehydrogenase, approx. 1100 U; citrate synthase, approx. 270 U
4. Three bottles 4 with lyophilizate acetyl-CoA synthetase, approx. 5 U each
5. Bottle 5 with acetic acid assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.) Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

1. Use solution of bottle 1 undiluted.
2. Dissolve contents of bottle 2 with 7 ml redist. water.
3. Use suspension of bottle 3 undiluted.
4. Dissolve contents of one bottle 4 with 0.25 ml redist. water.

Stability of reagents

Solution 1 is stable at 2-8°C (see pack label).

Bring solution 1 to 20-25°C before use.

The contents of bottle 2 are stable at 2-8°C (see pack label).

Solution 2 is stable for 4 weeks at 2-8°C, for 2 months at -15 to -25°C.

The contents of bottle 3 are stable at 2-8°C (see pack label).

The contents of the bottles 4 are stable at 2-8°C (see pack label).

Solution 4 is stable for 5 days at 2-8°C

Procedure

Wavelength³: 340 nm, Hg 365 nm or Hg 334 nm

Glass cuvette⁴: 1.00 cm light path

Temperature: 20-25°C

Final volume: 3.230 ml

Read against air (without a cuvette in the light path) or against water

Sample solution: 0.3-30 µg acetic acid/assay⁵ (in 0.100-2.000 ml sample volume)

Pipette into cuvettes	Blank	Sample
solution 1	1.000 ml	1.000 ml
solution 2	0.200 ml	0.200 ml
sample solution*	-	0.100 ml
redist. water	2.000 ml	1.900 ml
Mix** and read absorbances of the solutions (A ₀). Addition of:		
suspension 3	0.010 ml	0.010 ml
Mix** and read absorbances of the solutions (A ₁) after approx. 3 min. Start reaction by addition of:		
solution 4	0.020 ml	0.020 ml
Mix**, wait until the reaction has stopped (approx. 10-15 min) and read the absorbances of the solutions (A ₂). If the reaction has not stopped after 15 min, continue to read the absorbances at 2 min intervals until the absorbance increases constantly for 2 min.		

* Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

If the absorbance A₂ increases constantly, extrapolate the absorbance to the time of the addition of solution 4 (ACS) (see also pt. 7).

Determine the absorbance differences (A₁-A₀) and (A₂-A₀) for blank and sample.

With the equilibrium of the preceding indicator reaction, there is no linear (direct) proportionality between the measured absorbance difference and the acetic acid concentration.

The following formula, which should generally be used for preceding indicator reactions, serves to calculate the ΔA_{acetic acid} (see Ref. 1.2):

$$\Delta A_{\text{acetic acid}} = \left[(A_2 - A_0)_{\text{sample}} - \frac{(A_1 - A_0)_{\text{sample}}^2}{(A_2 - A_0)_{\text{sample}}} \right] - \left[(A_2 - A_0)_{\text{blank}} - \frac{(A_1 - A_0)_{\text{blank}}^2}{(A_2 - A_0)_{\text{blank}}} \right]$$

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt.4).

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of NADH at:
340 nm = 6.3 [l × mmol⁻¹ × cm⁻¹]
Hg 365 nm = 3.4 [l × mmol⁻¹ × cm⁻¹]
Hg 334 nm = 6.18 [l × mmol⁻¹ × cm⁻¹]

It follows for acetic acid:

$$c = \frac{3.230 \times 60.05}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{1.940}{\epsilon} \times \Delta A \text{ [g acetic acid/l sample solution]}$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{Content}_{\text{acetic acid}} = \frac{c_{\text{acetic acid}} \text{ [g/l sample solution]}}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

1 ACS, also known as acetate thiokinase

2 AMP = adenosine-5'-monophosphate

3 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.

4 If desired, disposable cuvettes may be used instead of glass cuvettes.

5 See instructions for performance of assay



1. Instructions for performance of assay

The amount of acetic acid present in the assay has to be between 0.6 µg and 30 µg (measurement at 365 nm) or 0.3 µg and 15 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield an acetic acid concentration between 0.06 and 0.3 g/l or 0.03 and 0.15 g/l, respectively.

Dilution table

Estimated amount of acetic acid per liter measurement at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 0.15 g	0.3 g	-	1
0.15-1.5 g	0.3-3.0 g	1 + 9	10
1.5-15 g	3.0-30 g	1 + 99	100
> 15 g	> 30 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) or the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml. The volume of water added must then be reduced so as to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

2. Technical information

2.1 In the determination of acetic acid (acid samples), care should be taken that, when carrying out sample preparation and handling the sample generally, the volatile analyte is not lost. This can be avoided by alkalinizing the sample; the resulting acetate is not volatile.

2.2 In carrying out the calculation, a clear indication should be given as to whether the results are to be given as acetic acid (molar mass 60.05 g/mol) or as acetate (molar mass 59.04 g/mol). (In enzymatic determinations, the acetate ion is measured.)

3. Specificity (Ref. 1)

The method is specific for acetic acid.

In the analysis of commercial acetic acid (glacial acid), results of approx. 100% have to be expected (when producing acetic acid solutions, the volatility of acetic acid has to be taken into consideration); in the analysis of commercial water-free sodium acetate, results of < 100% have to be expected because the substance absorbs moisture.

4. Sensitivity and detection limit (Ref. 1.3)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume $v = 2.000$ ml and measurement at 340 of an acetic acid concentration of approx. 0.1 mg/l sample solution (if $v = 0.100$ ml, this corresponds to 1.5 mg/l sample solution).

The detection limit of 0.15 mg/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume $v = 2.000$ ml.

5. Linearity

Linearity of the determination exists from 0.3 µg acetic acid/assay (0.15 mg acetic acid/l sample solution; sample volume $v = 2.000$ ml) to 30 µg acetic acid/assay (0.3 g acetic acid/l sample solution; sample volume $v = 0.100$ ml).

6. Precision

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of $v = 0.100$ ml and measurement at 340 nm, this corresponds to an acetic acid concentration of approx. 1.5-3 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.015-0.03 g/100 g can be expected.)

The following data have been published in the literature:

CV = 0.6-1.6 % acetic acid solutions

CV = 1.5-1.8 % white wine

CV = 1.7-2.1 % red wine

CV = 2.3-2.8 % yogurt

(Ref. 1.3)

Boiled finely minced pork sausage:

$x = 0.3$ g/100 g $r = 0.017$ g/100 g $s_{(r)} = \pm 0.006$ g/100 g

$R = 0.023$ g/100 g $s_{(R)} = \pm 0.008$ g/100 g

Tomato ketchup: $r = 0.05$ g/100 g $s_{(r)} = \pm 0.02$ g/100 g

$R = 0.07$ g/100 g $s_{(R)} = \pm 0.02$ g/100 g

Bread:

$x = 131.89$ mg/100 g $r = 7.53$ mg/100 g $s_{(r)} = \pm 2.66$ mg/100 g

$R = 21.12$ mg/100 g $s_{(R)} = \pm 7.46$ mg/100 g

$x = 204.55$ mg/100 g $r = 7.41$ mg/100 g $s_{(r)} = \pm 2.62$ mg/100 g

$R = 19.35$ mg/100 g $s_{(R)} = \pm 6.84$ mg/100 g

(Ref. 2.1)

7. Interference/sources of error

Esters of acetic acid may be saponified under assay conditions (examples: ethyl acetate in wine; acetyl salicylic acid in pharmaceuticals). Acetic acid formed by slow hydrolysis is responsible for creep reactions which have to be taken into consideration when calculating results (extrapolation of A_2 to the time of the addition of the sample solution).

8. Recognizing interference during the assay procedure

8.1 If the conversion of acetic acid has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

8.2 On completion of the reaction, the determination can be restarted by adding acetic acid or sodium acetate (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard

The reagents used in the determination of acetic acid contain hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulations 67/548 and 99/45 and subsequent alteration, supplementation and adaptation guidelines. Please refer to the safety data sheet or the labels of the affected vials for further information.

10. General information on sample preparation

In carrying out the assay:

Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml; Filter **turbid solutions**;

Degas **samples containing carbon dioxide** (e.g. by filtration);

Adjust **acid samples** to approx. pH 8-9 by adding sodium or potassium hydroxide solution;

Adjust **acid and weakly colored samples** to pH 8-9 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;

Treat **“strongly colored” samples** that are used undiluted or with a higher sample volume with activated charcoal or with polyamide or polyvinylpyrrolidone (PVPP), e.g. 1 g/100 ml;

Crush or homogenize **solid or semi-solid samples**, extract with water or dissolve in water and filter if necessary; resp. remove turbidities or dyestuffs by Carrez clarification;

Deproteinize **samples containing protein** with perchloric acid; alternatively clarify with Carrez reagents;

Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter; alternatively clarify with Carrez-solutions after the extraction with hot water.

Carrez clarification:

Pipette the liquid sample into a 100 ml volumetric flask containing approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferrocyanide), 85 mM = 3.60 g $K_4[Fe(CN)_6] \times 3 H_2O/100 ml$) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 7.20 g $ZnSO_4 \times 7 H_2O/100 ml$). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

11. Application examples

Determination of acetic acid in fruit juices

a) Fruit juices with a high acetic acid content (for example, in the range of approx. 0.3 g/l): Dilute the sample with water 1 + 1; use 0.100 ml for the assay.

b) Fruit juices with a low acetic acid content (less than approx. 0.02 g/l):

Decolorize **colored juices**:

Add 1% (w/v) activated charcoal to the sample, stir for approx. 30s and filter. Use 0.500 ml for the assay (take into account the altered sample volume *v* in the calculation).

In certain situations (when using a large sample volume), adjust **acid juices** to pH 8.

Determination of acetic acid in wine (Ref. 3.1)

Use 0.100 ml of white wine undiluted for the assay (this volume may be increased up to 2.000 ml, if necessary).

Use 0.100 ml of red wine containing about 0.2 g of acetic acid/l undiluted for the assay without decolorizing.

To red wine containing less than 0.1 g acetic acid/l add 1% (w/v) polyamide or PVPP, stir for approx. 1 min and filter. Adjust an aliquot volume of the largely decolorized sample to pH 8 (indicator paper) with sodium hydroxide (0.1 M), dilute with water to give double the volume. Use up to 2.000 ml, if necessary, for the assay (take into account the dilution and the altered sample volume *v* in the calculations).

High alcohol concentrations in the sample may delay the acetate reaction. The absorbances A_2 should therefore be read after 20 min.

Determination of acetic acid in vinegar

Dilute the sample according to the dilution table and use 0.100 ml for the assay.

Determination of acetic acid in sour dressings and sauces

Separate solids from the sample and place it into a refrigerator for 20 min to obtain separation of fat. Filter, adjust filtrate to 20-25°C and dilute according to the dilution table, if necessary.

Determination of acetic acid in beer (Ref. 3.4, 3.5)

To remove the carbonic acid stir approx. 5-10 ml of beer for 30s with a glass rod or filter. The largely CO₂-free sample is used for the assay without further dilution.

Determination of acetic acid in hard cheese

Accurately weigh approx. 2 g of ground cheese into a 100 ml volumetric flask, add about 70 ml water and incubate at approx. 60°C for 20 min. Shake flask from time to time. After cooling to 20-25°C, fill up to 100 ml with redist. water. For separation of fat, place the flask in a refrigerator for 20 min, filter, discard the first few ml of the filtrate. Use the clear solution, which may also be slightly opalescent, adjusted to 20-25°C, for the assay.

Determination of acetic acid in mayonnaise or yogurt

Accurately weigh approx. 5 g of sample into a 100 ml volumetric flask, add approx. 50 ml redist. water and heat for 20 min in a waterbath at 50-60°C; shake from time to time. After cooling to 20-25°C, fill up to 100 ml with redist. water. For separation of fat, place the mixture for 20 min in a refrigerator. Filter solution and use the clear or slightly turbid solution, adjusted to 20-25°C for the assay.

Determination of acetic acid in meat products

Accurately weigh approx. 10 g minced sausage and homogenize with 80 ml perchloric acid (1 M) for 10 min using a homogenizer (Ultra Turrax, or IKA mill), centrifuge, decant the supernatant, and filter. Discard the first few ml of the filtrate and pipette 20 ml into a beaker, adjust to pH 10.0 with potassium hydroxide (2 M); measure the volume of KOH. To obtain quantitative precipitation of the potassium perchlorate formed, place in an ice-bath or refrigerator for 20 min; filter. Use the clear solution diluted, if necessary (see dilution table), for the assay. When calculating the dilution, take into account the water content of the sample.

For calculating the content (in g/100 g) according to the above-mentioned formula (see calculation) the content of the sample in the sample solution is needed. When applying the above-mentioned sample preparation and considering the water content of the sample the weight of the sample is calculated according to the following formula:

$$\text{Weight}_{\text{sample}} = \frac{a \times 1000 \times d}{(b + a \times w) \times (d + e)} \text{ [g/l]}$$

It is:

- a: the weighed sample in g
- b: volume of perchloric acid in ml
- d: volume of the aliquot of supernatant in ml
- e: volume of KOH for neutralization in ml
- w: water content of the sample in (%; w/w)/100

1000: factor for g expressed in mg

(The specific gravity of water from the sample at 20-25°C is approx. 1 g/ml. It can be neglected for the calculation.)

12. Further applications

The method may also be used in the examination of paper, pharmaceuticals (e.g. infusion solutions, acetyl salicylic acid preparations), emulsifiers (after alkaline hydrolysis) and in research when analyzing biological samples.

For details of sampling, treatment and stability of the sample see Bergmeyer, H. U. & Möllering, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd ed., vol. 3, p. 1523-1525, Verlag Chemie, Weinheim/Academic Press, Inc. New York and London; Holz, G. & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd ed., vol. 3, p. 1530, Verlag Chemie, Weinheim/Academic Press, Inc. New York and London; Lundquist, F. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd ed., vol. 3, p. 1534-1535, Verlag Chemie, Weinheim/Academic Press, Inc. New York and London.

Determination of acetic acid in fermentation samples and cell culture media

Place the sample, after centrifugation, if necessary, in a water-bath of 80°C for 15 minutes (cover the tube because of the volatility of acetic acid) to stop enzymatic reactions. Centrifuge and use the supernatant, diluted according to the dilution table, if necessary, for the assay. (Alternatively, deproteinization can be carried out with perchloric acid. See the abovementioned examples.)

Homogenize gelatinous agar media with water and treat further as described.

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- 2.9 Europäische Norm / European Standard EN 12632 (April 1999) , Frucht- und Gemüsesäfte: Enzymatische Bestimmung des Gehaltes an Essigsäure (Acetat) - Spektralphotometrische Bestimmung von NAD (Fruit and vegetable juices - Enzymatic determination of acetic acid (acetate) content - NAD spectrometric method)
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Acetic acid assay control solution (Bottle 5)

Concentration: see bottle label

Acetic acid assay control solution is a stabilized aqueous solution of acetic acid. It serves as an assay control solution for the enzymatic determination of acetic acid in foodstuffs and other materials.

Application:

1. *Addition of acetic acid assay control solution to the assay mixture:*
Instead of sample solution the assay control solution is used for the assay.

2. *Restart of the reaction, quantitatively:*

After completion of the reaction with sample solution and measuring of A_2 , add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 20 min.). An increase of absorbance is observed.

A calculation of results is not possible because of the preceding equilibrium reaction with L-MDH (3).

3. Internal standard:

The assay control solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
solution 1	1.000 ml	1.000 ml	1.000 ml	1.000 ml
solution 2	0.200 ml	0.200 ml	0.200 ml	0.200 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
redist. water	2.000 ml	1.900 ml	1.900 ml	1.900 ml

Mix, and read absorbances of the solutions (A_0). Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample} + \text{standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100 [\%]$$



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